

Analysis of Staphylococcal Enterotoxin B by the Polyacrylamide Electrophoresis Technique

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The electrophoretic mobility of enterotoxin B was investigated through the use of the disc electrophoresis technique. Ideal patterns were developed with a 7.5% acrylamide gel system (pH 4.3). The toxin can be separated and identified from other complex proteins such as serum or suspect samples of foods by this technique. The technique can be used as an assay method for the toxin as well as to elucidate physical changes in the toxin due to temperature. The method should not be considered exclusive for enterotoxin B.

The principal objective of electrophoresis is to provide a means of separating molecules, thereby identifying and classifying them. If one molecule is so peculiar or specific that it can be differentiated from all others, it can also be used to identify the organism from which it comes. The present study deals with a very specific compound elaborated by certain microorganisms, staphylococcal enterotoxin B. Until recently, very little electrophoretic work has been done with enterotoxin B because the Tiselius (20) moving boundary method is cumbersome; most zone electrophoretic methods were found to be impractical, and known or "standard" enterotoxin was not available.

For optimum results, consideration must be given to the isoelectric point and molecular weight of the material being studied as well as to all conditions under which the run is to be made. Staphylococcal enterotoxin is no exception to these considerations.

Hibnick and Bergdoll (10) using the Tiselius technique reported the isoelectric point of enterotoxin at pH 8.6 and a molecular weight of $24,000 \pm 3,000$. More recently Wagman et al. (21) reported a molecular weight of 35,300. Bergdoll (2) reported unsuccessful attempts to fractionate enterotoxin with paper electrophoresis. Thatcher et al. (19) also used paper as a support medium and pointed out the ability of filter paper to absorb enterotoxin and their inability to elute enterotoxin from it. They also investigated the use of "gold-beater's skin," parchment, and nylon fabric and found them impractical. Therefore, most workers, Bergdoll and co-workers (2, 3), Casman and Bennett (4), Baird-Parker and

Joseph (1), and Dalidowicz et al. (6), have used starch as a support medium.

With the use of acrylamide-gel as a support medium, as introduced by Raymond and Weintraub (14), and the technique developed by Davis (7) and Ornstein (11), an ideal system became available for zone electrophoresis. Frea et al. (9) used disc electrophoresis and the method of Davis (7) in their studies on purification of staphylococcal enterotoxin B, but consideration was not given to basic parameters for optimum results. Their patterns and results are not ideal and should be considered only a first approach inasmuch as it would be difficult to obtain further information by their method. In the present study, consideration is given to optimum conditions for the electrophoretic analysis of staphylococcal enterotoxin, and these conditions are presented.

MATERIALS AND METHODS

Partially and highly purified staphylococcal enterotoxin B was kindly supplied by E. J. Schantz, Fort Detrick, and prepared by the method of Schantz et al. (17). The lyophilized enterotoxin was dissolved in sterile, distilled water to give a concentration of 5 mg/ml. Portions of this were added to the sample gel. Disc electrophoresis procedures and methods were those of Davis (7) and were based on the theory of Ornstein (11). Amounts of enterotoxin, as indicated in the figures, were added to the sample gel. On the basis of isoelectric point and molecular weight, the gel buffer system of Reisfeld et al. (16) for basic proteins was used for optimum results and is similar to the method of Denny et al. (8) for enterotoxin A. The method was first referred to by Schantz et al. (17) as work done by this author but not reported in detail at that time. A constant current of 5 ma per tube was applied for 30 min at room temperature, unless other-

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wise noted. Gels were removed from tubes under cold water and stained with 0.5% Amido Schwartz in 7% acetic acid for at least 1 hr; excess stain was removed electrophoretically in an alcohol-acetic acid bath consisting of 500 ml of methanol, 500 ml of water, and 100 ml of acetic acid. A fixed and stained disc gel pattern was used as template for cutting out sections of unstained gel for subsequent elution of toxin for testing biological activity by injecting monkeys on a micrograms per kilogram basis. Elution was carried out overnight in 0.5 ml of saline at 5 C, and microgram amounts were recovered.

Larger amounts (milligrams) of staphylococcal enterotoxin were recovered by using slabs of acrylamide by the method of Raymond et al. (13) and by using the elution-convection method of Raymond and Jordan (12). One milliliter of a 5 mg/ml solution of enterotoxin was layered in the premolded slot of the acrylamide slab. Electrophoresis was carried out in a formate buffer (pH 3.5) for 6 hr with 120 ma across the gel. Running tap water was used for cooling. Elution was carried out with the same buffer and coolant and a current of 112 ma for 5 hr.

A 2-ml amount of a 5 mg/ml solution of highly purified enterotoxin B was heated in a boiling water bath for 5 and 10 min. Samples (20 μ liter) were taken for electrophoretic analysis. Twenty-microliter samples of the highly purified enterotoxin were also heated for 5 min at various temperatures from 30 to 90 C and again used for electrophoretic analysis.

RESULTS AND DISCUSSION

Figure 1 represents a variety of different preparations of staphylococcal enterotoxin B run over a period of several years. Since individual tubes may have been run at different times under slightly different conditions of temperature, time, and buffer ionic strength, the known toxin band may be at different distances from the origin. When working with unknowns, a known toxin was always run for comparison of bands. As can be seen, the number of bands varies from one to five, with the exception of 13A, which contains no bands and thus no protein. Of the toxins tested and illustrated in Fig. 1, it would appear that D2, 21, and 23 are homogenous and contain no impurities and are, therefore, the most highly purified material tested. However, when higher concentrations of enterotoxin are used (including D2, 21, and 23), additional bands may be seen (Fig. 2). Sample SC (Fig. 1) contains a large amount of toxin but also many other bands. Other samples, as can be seen, contain various amounts of toxins and impurities or polymers of the toxin. To prove that the heavy band in the figures assumed to be enterotoxin was in fact enterotoxin, an electrophoretic run was made with the method stated above except that the time was increased to 55 min to achieve greater separation between

bands. One gel was stained and destained as rapidly as possible and then used as a template for cutting unstained gels. These small selected areas of cut gels were eluted, and the eluted material was injected intravenously into monkeys at a dose of 2.2 to 3.3 μ g/kg. Five of six monkeys gave positive responses. There were no deaths and no response in controls injected with eluates from control gels. Sufficient material from the lighter or minor bands could not be recovered from small disc gels. Thus, the question still remained as to whether the other bands were impurities or polymers of the enterotoxin.

To test further the biological activity of the major and more especially minor bands, the elution-convection method of Raymond and Jordan (12) was used with a larger quantity of enterotoxin. This is more of a preparative method in contrast to the analytical disc electrophoresis method. Sufficient material was obtained from one of the minor bands as well as of the heavy band to allow bioassay in monkeys. Three of three monkeys injected at a dose rate of 4 μ g/kg gave

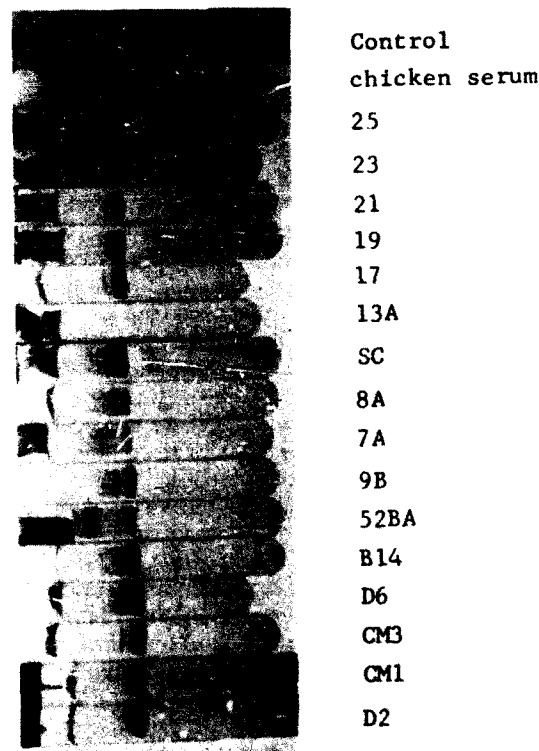


FIG. 1. Disc electrophoretic patterns of various partially and highly purified 20- μ liter samples of staphylococcal enterotoxin B. Anode and origin are to the left.

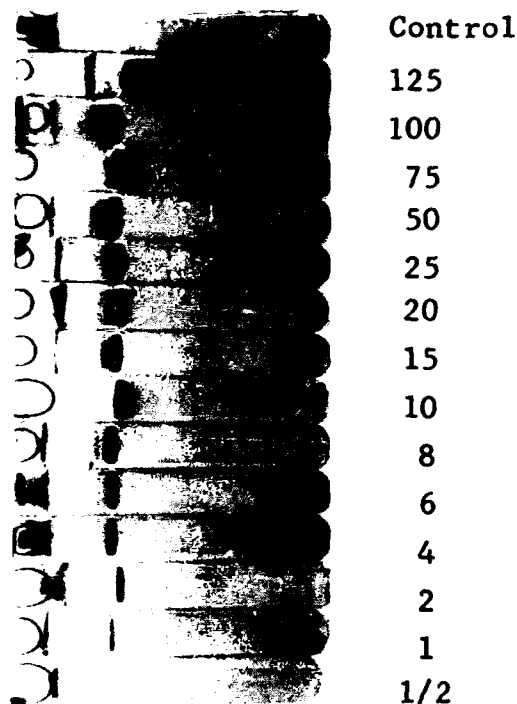


FIG. 2. Disc electrophoretic patterns from a single batch of staphylococcal enterotoxin B. Microgram amounts added to each gel are indicated in figure. Anode and origin are to the left.

positive responses from the major band, and two of three monkeys gave positive responses at the same dose level from the minor band. No deaths occurred in any monkeys. It is thus apparent that both the heavily and lightly stained bands contain biological activity even though they have different electrophoretic mobilities. Additional work should be done at higher dose levels to determine whether different biological activity is associated with the different bands alone or in combination. Since only emesis has been recorded in these few experiments with no deaths, the location of the lethal factor or cause of death in some cases as seen by other workers, Silverman et al. (18), has not been determined. The method can therefore be used to test for the presence or absence of toxin as well as for its purity and, hopefully, for a better understanding of the biological activity of the various factors of different electrophoretic mobilities.

In most cases, the appearance of impurities or polymers or, in any case, a nonhomogenous electrophoretic pattern depends on sample size. Thus, as material is diluted, impurities disappear. This is shown in Fig. 2. If a sample of 4 to 5 μ g is used, only one band is seen. At the 10- to 20- μ g level, another band becomes visible, and several are seen

at the 125- μ g level. It is evident from these results and the level of toxin used by Frea et al. (9) that they were not using optimum conditions for their electrophoretic procedure. When sharp or ideal patterns are obtained, as in Fig. 2, it is possible to use these patterns as a standard for comparison with unknown quantities for assay purposes.

Electrophoretic results can be obscured and misleading unless optimum conditions are used. Figure 3 further illustrates this point. In this experiment, tube 1 was run under optimum conditions given above, but tube 2 was run by the methods of Frea et al. (9), and the results were similar to theirs. It should also be noted that they failed to mention the band at the origin, as well as the faint band near the bottom of the tube. There are other diffuse bands present in their figure and, because of the conditions under which their run was made, none of the bands is sharp or ideal. These diffuse bands are important for they can contain biologically active material and can be better defined and isolated if optimum conditions are used. Their selection of the basic gel-buffer

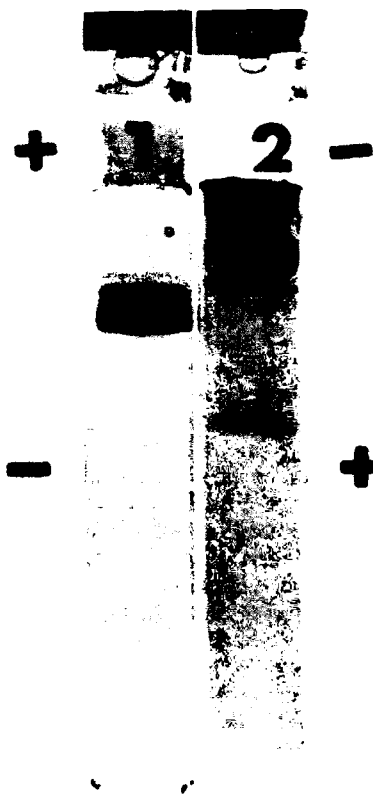


FIG. 3. Disc electrophoresis pattern of enterotoxin B run with different gel buffer system. Tube 1, pH 4.3 gel system; tube 2, pH 8.3 gel system. Both were run for 30 min. Origin at top in both cases.

system (pH 8.3) of Davis (7), Ornstein (11), and Gerloff (M.S. Thesis, Univ. of Wisconsin, 1963) when the isoelectric point of the toxin is 8.6 is a poor one because these two figures are too close for practical purposes. If the pH of the buffer system is not sufficiently different from the isoelectric point of the material being studied, either migration will not occur or it may not proceed in the desired direction. It is only possible to isolate and elute fractions when extremely sharp or discrete bands are present. Diffuse bands lead to overlapping of factors as well as reduction in concentration of material needed, and they are not improved with time since diffusion increases with time, especially at room temperature. Frey et al. (9) used 1.5 hr and 4 ma, according to the method of Gerloff (M.S. Thesis). Figure 4 shows how bands became more diffuse with longer electrophoretic runs and, in fact, can even be lost. The faint band one third down in the first tube becomes quite diffuse in the second tube and lost in successive tubes.

With proper conditions, it is possible to use the technique to further advantage. It was noted when enterotoxin was heated to boiling temperature the solution became turbid, and samples gave no bands after electrophoresis (Fig. 5). It can also be seen that the toxin band is partially changed after 5 min at 70°C and completely gone after 5 min at 80°C. This is also probably due to the toxin coming out of solution and forming a precipitate. Only



FIG. 4. Effect of time on disc electrophoretic patterns. Left to right, tubes were run for 30, 45, 60, 75, and 90 min, respectively, at pH 8.3.

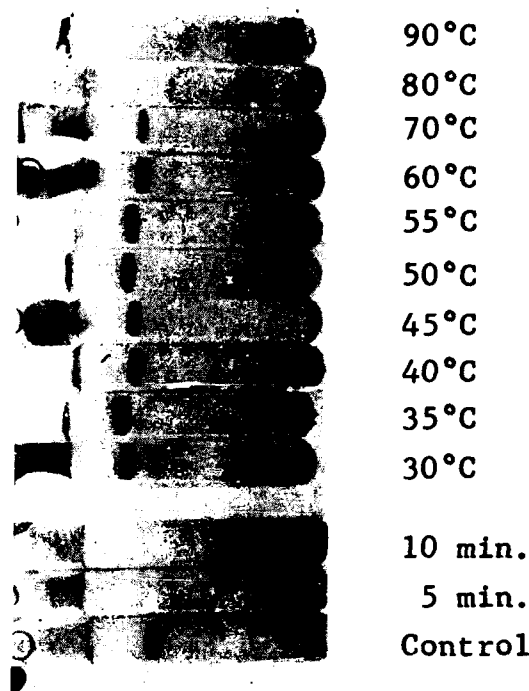


FIG. 5. Effect of temperature on enterotoxin B. Anode and origin to left. Control, not heated; 5 min represents sample boiled for 5 min, and 10 min represents sample boiled for 10 min. Tubes 30 through 90°C represent samples held for 5 min at temperature indicated.

overall changes as determined by electrophoresis were determined. The changes in biological activities have not been determined but should be. It would be of interest to determine whether biological activity is lost when staining quality of the protein is also lost. It is not believed that the toxin has been destroyed after 5 min at 80°C, since Read and Bradshaw (15) found that inactivation took place with longer exposure to higher temperature than used here. However, since there are changes in the electrophoretic bands at lower temperatures (Fig. 5), the method can be used to detect changes not previously considered. The technique can be used not only to follow purification of the toxin, but also to study inactivation or degradation such as by heat when coupled with assay in monkeys.

One of the greater practical problems in research on staphylococcal enterotoxin B has been the separation and identification of the toxin in the presence of other mixtures of proteins such as foods. To show the ease with which enterotoxin can be separated from protein, 5 mg of toxin per ml was mixed with 1 ml of chicken and 1 ml of beef serum, respectively, and 0.05 ml of this ma-

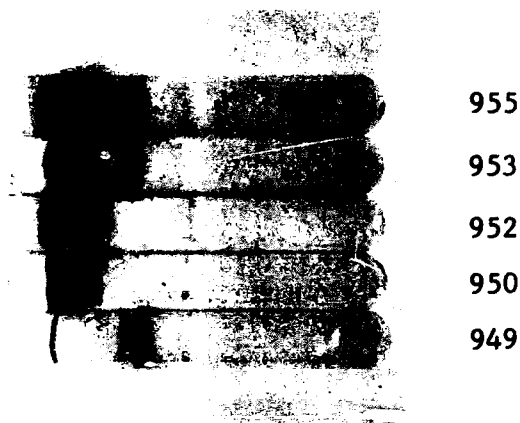


FIG. 6. Separation of enterotoxin B from chicken and beef serum. Tube 949, enterotoxin B; 950, chicken serum; 952, beef serum; 953, chicken serum and enterotoxin B; 955, beef serum and enterotoxin B. Anode and origin are to the left.

terial was added to sample gels and electrophoretic runs were made (Fig. 6). It should be noted that with the basic protein-gel buffer system of Reisfeld et al. (16), which is optimum for enterotoxin, serum fails to migrate into its usual 25 to 30 components and remains confined in an area close to the spacer gel, whereas the enterotoxin migrates through and beyond it. Preliminary studies were undertaken to show the usefulness of the technique in identifying enterotoxin in suspect food samples. A field sample of potato salad from a food poisoning episode in Indiana was tested by placing small amounts of the salad on the spacer gel and covering the sample with sample gel. The results indicate that several proteins were present because several bands were present. One very faint band was located in the same position as the major enterotoxin band when compared with a known enterotoxin control tube. As has been pointed out previously (Fig. 2), 1 μ g of enterotoxin in approximately 20 μ liters is about the lower limit of the technique. Thus, one problem in working with the field sample is concentration of the enterotoxin to a level that would be detectable in the small volume required for the test. This is not peculiar to the electrophoretic technique but applies to other techniques, as pointed out by Casman and Bennett (5), and can be overcome through their various extraction and concentration procedures.

It has been shown that, by proper selection of all conditions and methods, enterotoxin can be easily separated from complicated protein mix-

tures and that polyacrylamide disc electrophoresis can be used to advantage in the study of this toxin. Although only type B enterotoxin was used in this study, the principles involved indicate that the procedures and methods will hold for other toxin types and has been used by Denny et al. (8) in their studies on enterotoxin A.

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LITERATURE CITED

1. Baird-Parker, A. C., and R. L. Joseph. 1964. Fractionation of staphylococcal enterotoxin B. *Nature (London)* **202**: 570-571.
2. Bergdoll, M. S. 1956. The chemistry of staphylococcal enterotoxin. *Ann. N.Y. Acad. Sci.* **65**:139-143.
3. Bergdoll, M. S., H. Sugiyama, and G. M. Dack. 1959. Staphylococcal enterotoxin. I. Purification. *Arch. Biochem. Biophys.* **85**:62-69.
4. Casman, E. P., and R. W. Bennett. 1964. Production of antiserum for staphylococcal enterotoxin. *Appl. Microbiol.* **12**:363-369.
5. Casman, E. P., and R. W. Bennett. 1965. Detection of staphylococcal enterotoxin in food. *Appl. Microbiol.* **13**:181-189.
6. Dalidowicz, J. E., S. J. Silverman, E. J. Schantz, D. Stefanye, and L. Spero. 1966. Chemical and biological properties of reduced and alkylated staphylococcal enterotoxin B. *Biochemistry* **5**:2375-2381.
7. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**:404-427.
8. Denny, C. B., P. L. Tan, and C. W. Bohrer. 1966. Isolation and purification of enterotoxin A by polyacrylamide gel electrophoresis. *J. Environ. Health* **29**:222-230.
9. Frea, J. L., E. McCoy, and F. M. Strong. 1963. Purification of type B staphylococcal enterotoxin. *J. Bacteriol.* **86**:1308-1313.
10. Hibnick, H. E., and M. S. Bergdoll. 1959. Staphylococcal enterotoxin. II. Chemistry. *Arch. Biochem. Biophys.* **85**: 70-73.
11. Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. *Ann. N.Y. Acad. Sci.* **121**:321-349.
12. Raymond, S., and E. M. Jordan. 1966. Separation and recovery of proteins by elution convection. *Separ. Sci.* **1**:95-111.
13. Raymond, S., M. Nakamichi, and B. Aurell. 1962. Acrylamide gel as an electrophoresis medium. *Nature (London)* **195**:697-698.
14. Raymond, S., and L. Weintraub. 1959. Acrylamide gel as a supporting medium for zone electrophoresis. *Science* **130**:711.
15. Read, R. B., and J. G. Bradshaw. 1966. Thermal inactivation of staphylococcal enterotoxin B in Veronal buffer. *Appl. Microbiol.* **14**:130-132.
16. Reisfeld, R. A., U. J. Lewis, and D. E. Williams. 1962. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature (London)* **195**:281-283.
17. Schantz, E. J., W. G. Roessler, J. Wagman, L. Spero, D. A. Dunnery, and M. S. Bergdoll. 1965. Purification of staphylococcal enterotoxin B. *Biochemistry* **4**:1011-1016.
18. Silverman, S. J., D. A. Espeseth, and E. J. Schantz. 1969. Effect of formaldehyde on immunochemical and biological

- activity of staphylococcal enterotoxin B. *J. Bacteriol.* 98: 437-442.
19. Thatcher, F. S., B. H. Matheson, and W. R. Simon. 1955. Studies with staphylococcal toxins. *Can. J. Microbiol.* 1: 401-411.
- Tiselius, A. 1937. A new apparatus for electrophoretic analysis of colloidal mixtures. *Trans. Faraday Soc.* 33:524-531.
21. Wagman, J., R. C. Edwards, and E. J. Schantz. 1965. Molecular size, homogeneity, and hydrodynamic properties of purified staphylococcal enterotoxin B. *Biochemistry* 4: 1017-1023.